ONLINE METHODS

Antagomir synthesis. Antagomirs were designed and synthesized as described previously[28].

The antagomir-10b sequence was:

5'-oCsoAsoCoAoAoAoUoUoCoGoGoUoUoCoUoAoCoAoGoGsoGsoUsoAs-Chol-3'.

The antagomir-10b_mm sequence was:

5'-oAsoCsoGoAoUoAoAoAoCoGoGoUoUoGoUoCoUoAoCoGsoUsoCsoAs-Chol-3'.

All nucleotides used in synthesis were 2'-*O*-Me-modified (Subscript "o"). Subscript "s" represents a phosphorothioate linkage. "Chol" represents cholesterol linked through a hydroxyprolinol linkage.

Cell line. The 4T1 cell line was purchased from ATCC and cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For antagomir treatment, 4T1 cells were incubated with 50 μg/ml antagomir-10b.

RNA interference. SMARTpool Hoxd10 (Dharmacon) represents four pooled SMART-selected siRNA duplexes that target mouse *Hoxd10*. 4T1 Cells were transfected with 200 nM of the *Hoxd10* siRNA or control oligonucleotides using the Oligofectamine reagent (Invitrogen).

miRNA sponge. The miR-10b sponge was constructed using a method modified from previous reports[17, 37]: annealed oligonucleotides for tandem miR-10b binding sites were ligated into the pcDNA5-CMV-d2eGFP vector (Invitrogen) digested with XhoI and ApaI. The *gfp* mRNA along with the miR-10b sponge sequence in the 3'UTR was then subcloned into the pBabe-puro vector digested with BamHI and SalI. The control sponge was described previously[17, 37].

RNA isolation and miRNA quantification. Total RNA, inclusive of the small RNA fraction, was extracted from cultured cells or homogenized mouse tissues using the mirVana miRNA Isolation Kit (Ambion). Quantification of the mature form of miRNAs was performed using the TaqMan MicroRNA Assay Kit, according to the manufacturer's instructions (Applied Biosystems). The U6 small nuclear RNA was used as an internal control.

Real-time RT-PCR of mRNAs. Total RNA was reverse transcribed with an iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA was used for PCR using the SYBR-Green Master PCR Mix (Applied Biosystems) in triplicates. PCR and data collection were performed on iCycler (Bio-Rad). The expression levels of samples were determined using the standard curve method. Data were normalized to an internal control *Gapdh*. Primer sequences for mouse *Hoxd10* are: forward – AACAGATCTTGTCGAATAGAGCAAC; reverse – GGGCTGTTATTGTACTCTTGGGTTT. Primer sequences for *Gapdh* were described previously[19].

Immunoblotting. Western blot analysis was performed as described previously[12], using antibodies against Hoxd10 (sc-33004, 1:200, Santa Cruz) and Hsp90 (H38220-150, 1:3000, BD Biosciences).

***In vitro* growth curves.** Growth curves were determined as described previously[12].

In vitro **migration and invasion assays.** Transwell migration assays and Matrigel invasion assays were performed as described previously[12].

Metastasis studies. Animal experiments were performed in accordance with a protocol approved by the MIT committee on Animal Care. Surgery (mammary fat pad implantation), necropsy, and histological analysis were performed essentially as described[12]. Six- to eight-week-old female Balb/c mice (from Jackson Laboratory) were used for implantation of 4T1 cells (one million cells per mouse). The antagomir treatment started two days after orthotopic tumor cell implantation: PBS, antagomir-10b, or antagomir-10b_mm (50 mg/kg) was injected via tail vein, twice weekly for three weeks. All mice were requested to be euthanized four weeks after tumor cell implantation due to large primary tumor burdens. The mammary tumors were removed and weighed. Lung metastases were examined and counted under a dissecting microscope equipped with bright field imaging. Tissue samples were fixed in 10% buffered formalin for overnight, followed by wash with PBS and transfer to 70% ethanol, and then embedded in paraffin, sectioned, and stained with hematoxylin & eosin. To assess the antagomir-10b effect on late stages of the metastatic process, Balb/c female mice were implanted with 5×10^5 4T1 cells via tail vein, and antagomir-10b treatment started on day 6 post tumor cell transplantation, with the same dose and frequency as used in the orthotopic experiments; mice were moribund on day 19 due to lung metastases and were euthanized.

Toxicity assessment. Balb/c mice, five animals per group, were dosed intravenously with PBS or 50 mg/kg of antagomir-10b or antagomir-10b_mm twice a week for three weeks (6 doses). Body weight was determined twice a week during the study. 24 hours after the last dose, animals

were euthanized and tissues were harvested. Blood was collected by retro-orbital bleeding just before euthanasia. An aliquot of whole blood was sent to Molecular Diagnostic Services, Inc., Rabbit & Rodent Diagnostic Associates (RRDA). The remainder was collected in EDTA-treated tubes. Plasma was obtained by removing blood cells through centrifugation and was run on an Olympus Bioanalyzer in order to determine blood chemistry values. Liver sections were examined by a pathologist (J.T-F.) for all possible pathological conditions.

Statistical analysis. Data are presented as mean ± s.e.m. Student's *t* Test (two-tailed) was used to compare two groups for independent samples, assuming equal variances on all experimental data sets. Spearman's Rank Correlation Test was used to test correlation between a sequence of pairs of values; *R* stands for the correlation coefficient (a measure of the strength of the correlation) that varies from –1 (perfect inverse correlation) through 0 (no correlation) to +1 (perfect positive correlation).